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Origin of the human alcohol dehydrogenase system: Implications from the structure and properties of the octopus protein

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ABSTRACT **In contrast to the multiplicity of alcohol dehydrogenase in vertebrates, a class III type of the enzyme [i.e., a glutathione-dependent formaldehyde dehydrogenase; formaldehyde;NAD$^+$ oxidoreductase (glutathione-formylating), EC 1.2.1.1.] is the only form detectable in appreciable yield in octopus. It is enzymatically and structurally highly similar to the human class III enzyme, with limited overall residue differences (26%) and only a few conservative residue exchanges at the substrate and coenzyme pockets, reflecting "constant" characteristics of this class over wide time periods. It is distinct from the ethanol-active "variable" class I type of the enzyme (i.e., classical liver alcohol dehydrogenase; alcohol:NAD$^+$ oxidoreductase, EC 1.1.1.1). The residue conservation of class III is also spaced differently from that of class I but is typical of that of proteins in general, emphasizing that class I, with divergence at three functional segments, is the form with deviating properties. In spite of the conservation in class III, surface charges differ considerably. The apparent absence of a class I enzyme in octopus and the constant nature of the class III enzyme support the concept of a duplicative origin of the class I line from the ancient class III form. Still more distant relationships define further enzyme lines that have subunits with other properties.**

Previous analyses of alcohol dehydrogenases have traced a distant gene duplication (1) and an apparent genesis of the ethanol-active class I type (alcohol:NAD$^+$ oxidoreductase, EC 1.1.1.1) from the formaldehyde/glutathione-active class III type [formaldehyde:NAD$^+$ oxidoreductase (glutathione-formylating), EC 1.2.1.1] (2). Initial estimates from the divergence of the human/amphibian molecules tentatively suggested that the gene duplication occurred during early vertebrate evolution, about 450 million years ago (1). However, the similarity of the yeast and plant enzymes to the animal forms is then unexplained and, provided the dating holds true, leads to the conclusion that activity toward ethanol has evolved repeatedly (3). If so, the metabolic roles of the system of multiple alcohol dehydrogenases in humans may be complex.

To establish these relationships further, we decided to trace the original functions of the class I and III types of alcohol dehydrogenase by screening for these enzymes in invertebrates (4), possibly linking them to the vertebrate enzymes. We find evidence for just one alcohol dehydrogenase in cephalopods. Analysis of the enzyme reveals a single homogeneous protein, functionally and structurally closely related to the class III human enzyme. This finding indicates a basic metabolic need for glutathione-dependent formaldehyde dehydrogenase as an original function of the alcohol dehydrogenase system. The results also emphasize that some animals do not require any ethanol dehydrogenase activity, supporting the concept of multiple evolution of activity toward ethanol and highlighting the complex divergence of the mammalian enzyme system.

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MATERIALS AND METHODS

Protein and Enzymatic Analysis. Alcohol dehydrogenase was purified from gills, salivary glands, and branquial heart of octopus, monitoring octanol dehydrogenase activity (4). The enzyme was purified through ion-exchange chromatography on DEAE-Sepharose and affinity chromatography on Blue-Sepharose (4). Conditions for starch gel electrophoresis, activity staining, SDS/polyacrylamide gel electrophoresis, and protein staining have been given (4). For enzymatic analysis, kinetic parameters were determined at pH 7.5 and pH 10 as described (4).

Structural Analysis. For structural analysis, the protein was carboxymethylated by treatment with neutralized ^{14}C-labeled iodoacetic acid (Amersham; dilution to 2400 cpm/nmol; 1.5 μmol/mg of protein; for 1.5 hr at 37°C under N_2 in the dark) in 6 M guanidine hydrochloride/0.4 M Tris/2 mM EDTA, pH 8.1, after reduction with dithiothreitol (70 μg/mg of protein at 37°C for 2 hr). Reagents and buffers were removed by exclusion chromatography on PD-10 or by HPLC on Ultropac TSK G2000PW (7.5 × 600 mm; LKB). The carboxymethylated protein was digested in separate batches with *Achromobacter* lysine-specific protease (Wako Chemicals, Neuss, F.R.G.), *Pseudomonas* aspartic acid-specific protease (Boehringer Mannheim), and *Staphylococcus* glutamic acid-specific protease (Miles). The digests were fractionated by reverse-phase HPLC on Vydac C_8 and TSK ODS-120T C_{18} as described (5). Peptides obtained were analyzed for amino acid sequence by degradations in solid-phase (MilliGen Prosequencer 6600, with on-line analyzer) and gas-phase (ABI 477A, with on-line 120A analyzer) sequencers. Total compositions were determined with a Pharmacia LKB 4151 Alpha plus analyzer after acid hydrolysis for 24 hr at 110°C in evacuated tubes with 6 M HCl/0.5% phenol. High-sensitivity analysis utilized phenylthiocarbamyl derivatives of the amino acids (6). The N-terminal fragment was identified by electrospray mass spectrometry on a Perkin–Elmer Sciex API-III instrument (7).

Comparisons. The primary structure was compared with corresponding forms of yeast (8, 9), piscine (2), and mammalian (10) enzymes. Interactions in relation to the three-dimensional structure of class III alcohol dehydrogenase (11) were evaluated after alignments.

RESULTS

Purification and Enzymatic Properties. Octopus alcohol dehydrogenase was purified by monitoring octanol dehydrogenase activity in a two-step chromatographic procedure utilizing DEAE-Sepharose and Blue-Sepharose. Only one fraction with activity was detected, which yielded several

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bands with closely adjacent mobility on activity staining after starch gel electrophoresis and one band upon SDS/polyacrylamide gel electrophoresis that corresponded to a subunit of M_r about 40,000. The activity profile was highly positive for formaldehyde/glutathione (specific activity, 3.7 units/mg with 1 mM formaldehyde/1 mM glutathione, pH 8.0), high for octanol (3.3 units/mg with 1 mM octanol, pH 10.0), and low for ethanol (0.2 unit/mg with 0.1 M ethanol, pH 10.0). Kinetic parameters for the pure enzyme have been determined (4). Those with formaldehyde/glutathione, octanol, ethanol, and NAD^+ are summarized in Table 1 and compared with corresponding values for the human class III enzyme (15, 16). As shown, the enzymatic parameters of the human and the octopus forms are almost indistinguishable. Furthermore, properties are in accord with those exhibited by cephalopod alcohol dehydrogenases in general (4) and suggest that octopus has only one alcohol dehydrogenase that is expressed in appreciable yield and that this is of the class III type.

Primary Structure. Attempts at direct sequence analysis did not produce any results, indicating that the N terminus of octopus alcohol dehydrogenase is blocked like those of other animal alcohol dehydrogenases (18). The structure was determined by analysis of HPLC-purified peptides from four sets of proteolytic treatments, with lysine-specific, aspartic acid-specific, and glutamic acid-specific proteases, and CNBr, respectively. Nearly all peptides from the lysine-specific cleavage were recovered and analyzed as well as a considerable fraction of the glutamic acid-specific cleavage products, whereas only some of the aspartic acid-specific and CNBr fragments were recovered pure. The large and incompletely separated CNBr fragments were used for enzymatic redigestions. The combined results give a continuous amino acid sequence of 378 residues (Fig. 1).

The nature of the residues at a few positions (shown by lowercase letters in Fig. 1) is considered somewhat tentative, because of analytical problems (positions 3, 4, 138, and 353). However, also for these positions the structure given is in agreement with the majority of the results. The protein subunit was deduced to end with Glu-378 from recovery in a lysine-specific cleavage peptide. However, the possibility of C-terminal heterogeneity or truncation is not excluded. The blocked N terminus was established as threonine by compositional analysis of the lysine-specific cleavage peptide K1 and the aspartic acid-specific cleavage fragment MD1 (Fig. 1). K1 was also analyzed by electrospray mass spectrometry, showing the mass to be 972.5, compatible with acetyl as the blocking group (theoretical peptide mass then 972.54).

Conformation and Functional Properties. Alignment of the structure obtained shows a strict homology with human class III alcohol dehydrogenase. No insertions or deletions are required for maximal fit, except for different lengths at both ends (three extra residues at the N terminus and two at the C terminus). The overall residue identity is 74% toward the human class III alcohol dehydrogenase (279 of 378 residues). Considering the evolutionary distance from octopus to mammals, this extent of identity is extensive and corresponds to just intravertebrate differences for the class I enzyme (19).

Critical patterns—like glycine residues at reverse turns, proline distributions, and functionally active cysteine, histidine, and aspartic acid residues—are all conserved. This includes the three ligands to the active-site zinc atom [Cys-46, His-67, and Cys-174 in the mammalian class I enzymes (13, 14)] corresponding to Cys-47, His-69, and Cys-176 in Fig. 1; the four ligands to the second zinc atom (Cys-97, Cys-100, Cys-103, and Cys-111) corresponding to Cys-99, Cys-102, Cys-105, and Cys-113 in Fig. 1; the coenzyme ribose-interacting Asp-223 (12, 13) corresponding to Asp-225 in Fig. 1; and the coenzyme pyrophosphate-interacting pattern typical of nucleotide-binding proteins [Gly-Xaa-Gly-Xaa-Xaa-Gly (20–23)] corresponding to the glycine residues at positions 201, 203, and 206 in Fig. 1. In fact, only three exchanges are noticed for all of the 36 residues deduced to be in interactions with the coenzyme or substrate in the mammalian class I or III enzymes (11–13), and these three constitute fairly minor exchanges (Table 1) encountered in other enzyme variants before. These findings and the fact that still more distantly related alcohol dehydrogenases are possible to model into the three-dimensional structure of the mammalian enzymes (11, 24) suggest that the amino acid sequence determined fits into an essentially unaltered conformation relative to the class III human enzyme (11). The structural data prove the close functional similarity between human and octopus class III alcohol dehydrogenases, accounting for the enzymatic properties determined (Table 1).

Special Properties and Surface Charge. In spite of all similarities, the octopus enzyme has unique properties. Thus, the molecule is charged differently from the human/mammalian class III forms, with a more positive net charge [+2 per subunit versus the human form (Table 2)], and the molecule has a more cathodic migration upon electrophoresis [with pI of 6.9, in contrast to 6.4 for the human class III enzyme (15)]. These residue exchanges are positioned superficially, and the species variants in general distributed evenly in both domains of the subunit (Fig. 2). This pattern of differences constitutes a further distinction toward the class I enzyme (see *Discussion*) but is a pattern supporting a well-conserved ancient function.

Table 1. Enzymatic properties of class III alcohol dehydrogenases and corresponding residue exchanges at the substrate and coenzyme-binding pockets between the human and octopus enzymes

Property	Human	Octopus
Activity (pH 10.0)		
Ethanol		
K_m, mM	NS	NS
k_{cat}, min^{-1}	NS	NS
k_{cat}/K_m, $mM^{-1}\cdot min^{-1}$	0.045	0.086
Octanol		
K_m, mM	1.2	1.45
k_{cat}, min^{-1}	223	710
k_{cat}/K_m, $mM^{-1}\cdot min^{-1}$	185	490
S-Hydroxymethylglutathione		
K_m, mM	0.004	0.0015
k_{cat}, min^{-1}	200	300
k_{cat}/K_m, $mM^{-1}\cdot min^{-1}$	50,000	200,000
NAD^+		
K_m, mM	0.025	0.035
Structural differences		
Substrate interactions*		
Position 140	Tyr	Phe
Coenzyme interactions†		
Position 202	Gly	Ala
Position 222	Val	Ile

The alignment does not require introduction of gaps and is obvious from the structure of Fig. 1 and the replacements in Table 2. Positions for interactions from ref. 12 are supplemented by refs. 11, 13, and 14. As shown, enzymatic properties are indistinguishable, and residue exchanges are minimal. Values are from refs. 15 and 16 for the human enzyme and from ref. 4 for the octopus enzyme. *S*-Hydroxymethylglutathione is the product of the spontaneous conjugation of formaldehyde and glutathione and is considered the real substrate for formaldehyde dehydrogenase activity of class III (17). NS, no saturation.

*Positions 48, 57, 67, 93, 116, 141, 294, 306 and 318 were unaltered.

†Positions 47, 48, 51, 201, 223, 224, 228, 269, 271, 369, and 14 positions more (12) were unaltered.

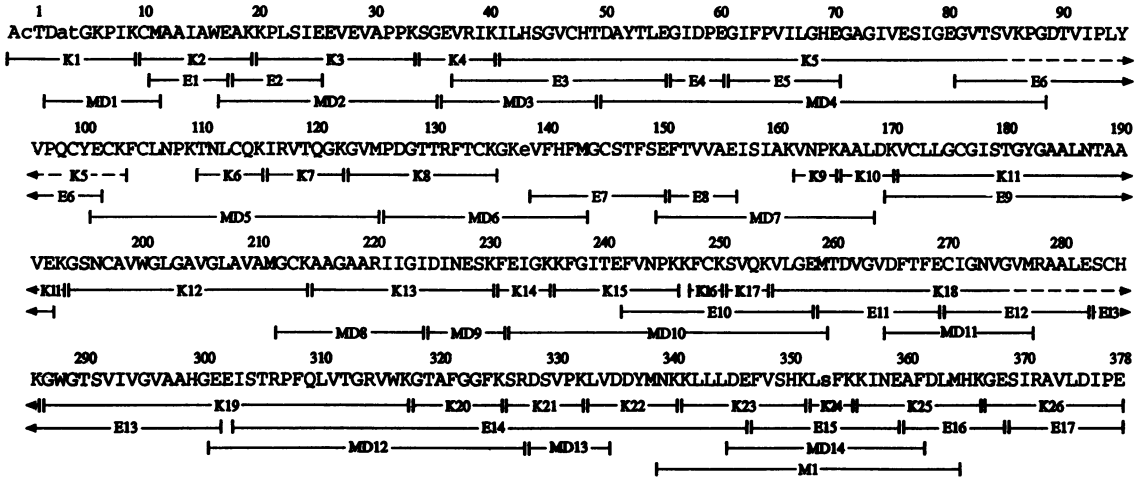

FIG. 1. Primary structure of octopus alcohol dehydrogenase and positions of peptides analyzed. Letters indicate the cleavage method for peptide generation, with K for lysine-specific protease, D for aspartic acid-specific protease, E for glutamic acid-specific protease, and M for CNBr. Noncapital letters indicate some ambiguity (see text). Continuous lines show those parts of each peptide analyzed by sequencer degradations (or, for K1, mass spectrometry), and dashed lines show the remaining parts.

DISCUSSION

Constant Class III, with Properties Typical of a Functionally Conserved Protein. The results show that the octopus alcohol dehydrogenase is a typical class III form. Both functionally and structurally, it has properties similar to that particular class of the five classes presently distinguished in the human alcohol dehydrogenase system. A consistent pattern emerges: the class III structure is present in prokaryotes (25), yeasts (8, 9), invertebrates (this work), and both early vertebrates (2) and mammals (10), as is also the class III activity (17). Throughout, residue differences are limited and proportional to the separation of the lines, showing an ancient and evolutionarily constant enzyme with glutathione-dependent formaldehyde dehydrogenase activity.

Residue exchanges that do occur resemble those in species variants of enzymes in general—i.e., are largely outside the active site and other functional regions. This is clearly visible in Fig. 2, showing few or no exchanges in the region around the active-site zinc atom (center of Fig. 2), the area of subunit interactions (two noncharged exchanges in Fig. 2), and the segment around the second zinc atom (devoid of exchanges). Furthermore, although exchanges involving charged residues affect one third of all positions (Table 2), all such exchanges are spaced around the surface of the subunit and largely those surfaces not participating in subunit interactions (Fig. 2).

"Variable" Class I, with Atypical Properties. The properties of class III, discussed above, are all in marked contrast to those of the ethanol-active class I alcohol dehydrogenase. This form is absent in octopus, at least in amounts now discernible, down to about the 1% level of the class III form. Furthermore, the class I form is more variable—the extent of variability now detected for human/octopus class III (26%) corresponds to just the divergence between the mammalian/avian class I proteins (19), whereas in bony fish, the class I form is already more closely related overall to class III than to class I (2).

The differences in the patterns of residue exchanges are even more pronounced. Noticeably, the differences in the class I enzyme are concentrated to those regions that are constant in the class III line. Thus, the class I enzymes exhibit maximal divergence at three segments close to the

Table 2. Exchanges between the octopus and human class III alcohol dehydrogenases that alter charges

	Amino acid residue				Amino acid residue		
Position	Human	Octopus	Difference*	Position	Human	Octopus	Difference
2	—	D	−1	236	E	K	+2
6	E	K	+2	246	Q	K	+1
11	K	M	−1	247	D	K	+2
19	G	K	+1	254	E	K	+2
35	H	G	−1	275	K	G	−1
43	A	H	+1	299	S	H	+1
55	S	E	−1	327	V	R	+1
84	K	S	−1	335	S	D	−1
138	T	E	−1	343	K	L	−1
163	D	N	+1	351	N	K	+1
165	L	K	+1	355	D	K	+2
190	K	A	−1	356	E	K	+2
193	P	K	+1	359	K	E	−2
228	K	E	−2	366	S	K	+1
229	D	S	+1	368	K	E	−2
232	A	E	−1	375	K	D	−2
233	R	I	−1	378	—	E	−1

Positional numbers refer to the octopus enzyme, as given in Fig. 1.
*Octopus versus human.

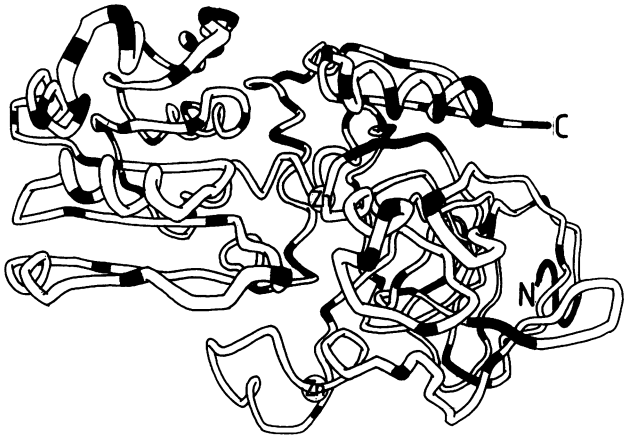

FIG. 2. Positions of all residue changes between octopus and human class III alcohol dehydrogenase. The subunit conformation is that deduced for the human class III protein in relation to the class I enzyme (11). N- and C-terminal extensions of the octopus protein chain versus the human class I protein by one and two residues, respectively, are drawn as straight extensions. Exchanges altering charges are solid black, and those not altering charges are stippled. As shown, exchanges are underrepresented in three regions—the active site (center), the area of subunit interactions (bottom left), and the second zinc atom (bottom center/right)—but are distributed evenly between the two domains.

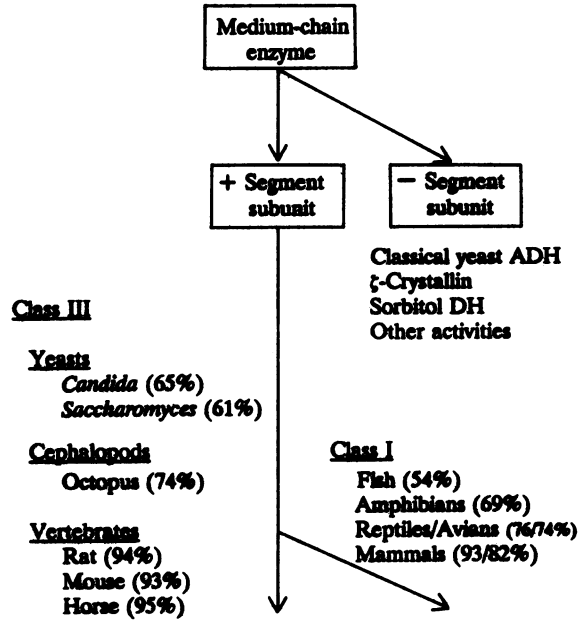

FIG. 3. Schematic relationships among characterized medium-chain alcohol dehydrogenases. "+ Segment" and "− Segment" indicate the two lines leading to the two types of alcohol dehydrogenase—with and without an internal segment (positions 119–138 or 119–139; ref. 28) and associated with different quaternary structures (dimeric and tetrameric, respectively). Values within parentheses give residue identities (in per cent) between each enzyme and the corresponding human form. Alignments of class I enzymes are given in refs. 1, 5, and 19, and those between the class III enzymes are in refs. 8–10. DH, dehydrogenase; ADH, alcohol DH. Novel features are the separate levels of divergence, with class III a parent form, also for the plant enzymes (not depicted, but present-day maize ADH exhibiting 54% residue identity to human class III, versus only 49% to human class I), and the still earlier separation of "+ segment" and "− segment" lines, making yeast ADH occurring in both lines (*Saccharomyces* class III and classical yeast ADH, respectively).

active site, around the second zinc atom, and in the segment of subunit interactions—a pattern that has been noticed as a peculiar property of that protein (26). This notion is now strongly supported as peculiar and is in marked contrast with the pattern of the class III proteins in Fig. 2. Therefore, the results demonstrate the different nature of the two classes of alcohol dehydrogenase, with the properties of class I deviating from those of proteins in general by exhibiting variability in functional segments, while class III exhibits properties typical of functionally conserved proteins.

Enzyme Relationships and Functions. The general presence of class III alcohol dehydrogenase in living organisms and the absence of class I in cephalopods, the separate distributions of the residue exchanges within class III (Fig. 2) versus those within class I (26), and the hybrid properties of the class I piscine alcohol dehydrogenase (functionally class I, but structurally overall closer to class III) suggest that the class I line has originated from the class III line through a gene duplication (Fig. 3). Mutational differences have then given rise to the class I form, apparently first acquiring the enzymatic properties [already in fish (2)] in a structure that overall is more related to present day mammalian class III than to mammalian class I. Further accumulation of differences later apparently also have given the class I form its distinct structural entity (now observable in the postpiscine lines). However, class I still exhibits great variability, repeated isozyme formations, and accumulation of species differences at important segments. In total, the class I pattern suggests an enzyme susceptible to functional divergence. The timing of the class I/III gene duplication is difficult to establish, since early changes after duplication may well be nonlinear, but a timing at about early vertebrate evolution (1) still appears compatible with all data. In any event, class I is far from equally ancient and constant as class III (Fig. 3).

This verification of the class I/III differences clarifies also still earlier events. Thus, the traditional yeast enzyme, although ethanol-active, exhibits a third type of structural pattern. It lacks an internal segment, present in both class I and class III alcohol dehydrogenases, and is far more divergent, with residue identities only at the 25% level (27, 28). These relationships suggest the presence of an early divergence with two lines, one having and the other lacking the extra segment (Fig. 3). Plant alcohol dehydrogenases, also well known, have the internal segment and appear to be derived from the segment-positive line. Although their topography is still difficult to discern, they show several class III rather than class I features. Relationships to both classes are distant, but the plant enzymes exhibit a greater identity toward class III than toward class I (54% for the maize enzyme versus the human class III form but only 49% versus the human class I form). Consequently, an early divergence from the ancient class III line seems possible also for the plant ethanol-active alcohol dehydrogenases. The properties of the yeast and plant enzymes therefore now fit into an evolutionary scheme, not contradicting the pattern from the animal lines (Fig. 3). Instead, the ethanol type of substrate specificity in higher plants and vertebrates may reflect convergence toward that activity rather than a divergence from such an original activity.

Functionally, the present pattern indicates that the original form of alcohol dehydrogenase is of the class III type, which is associated with glutathione-dependent pathways. This suggests that alcohol dehydrogenases take part in metabolic defense functions similar to those of glutathione transferases. Noticeably, both these protein families constitute large enzyme systems with repeated gene duplications and divergence at different levels (29). Nevertheless, the activity toward ethanol, other shortchain primary alcohols, and nonconjugated aldehydes does not appear to be essential to life, since it seems not to be of ancient origin (Fig. 3) but appears to have arisen separately (3). Alcohol dehydrogenase-negative animal strains detected previously (30) lack class I

but appear to fulfill that function through other classes (31), probably class IV (32, 33). Therefore, cephalopods are the first detected group of animals that lack the ethanol dehydrogenase activity (4). The class I alcohol dehydrogenase function appears more recent, variable, and acquired repeatedly than the ancient class III defense function with specificity for conjugated formaldehyde. The continuous divergence of class I may explain why it has been difficult to ascribe just a single function to liver alcohol dehydrogenase. Nevertheless, such a metabolic function may also exist in addition to the variable patterns and may be anchored by the further isozyme divergence in vertebrate lines.

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